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Composite, large spirochetes from microbial mats: Spirochete structure review

(round bodies/spirochete membranous bodies/spirochete life history/variable-diameter spirochetes/*Spirosymplokos*)

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ABSTRACT **Phenomena previously unknown in free-living spirochetes are reported: large-sized cells with variable diameter (length to 100 μm, width between 0.4 and 3.0 μm), composite structure (smaller spirochetes inside larger ones), and positive phototropic behavior. These bacteria, *Spirosymplokos*, are compared with all other spirochete genera. The large spirochete, grown in mixed culture, was studied live and by transmission EM. The protoplasmic cylinder was replete with spherical granules 20–32 nm in diameter, and three to six periplasmic 26-nm flagella were inserted subterminally. Comparably granulated and flagellated small spirochetes were located inside the protoplasmic cylinder and in the periplasm of the large ones. When exposed to air, movement became erratic, protoplasmic cylinders retracted to lie folded inside the outer membrane, and refractile membranous structures formed. From one to four structures per still-moving spirochete were seen. *Spirosymplokos* was enriched from laboratory samples exposed to oxygen-rich and desiccating, but not dry, conditions for at least 4 mo after removal of microbial mat from the field.**

Spirochetes, microscopic "wriggly hairs," were confused with trypanosomes, other protists, and bacteria (1‖, 2). Although first named by C. S. Ehrenberg in the 1830s, not until ultrastructural studies were undertaken (3, 4) was Noguchi's claim that spirochetes are bacteria demonstrated unequivocally. A unified group of highly motile prokaryotes, they bear their flagella in the periplasm—i.e., beneath the outer membrane (5). Each helically shaped cell minimally has 2 flagella (e.g., *Spirochaeta*) and maximally >300 [*Cristispira* (6)]. Arranged symmetrically, the flagella tend to overlap. All spirochetes are placed in a single phylum, Spirochaetes (7), of the Kingdom Procaryotae or Monera (8). They are described by the expression *n*:2*n*:*n*, where *n* is the number of flagella at a terminus (Fig. 1). When the flagella are too short to overlap, as in *Leptospira* or *Treponema phagedenis*, the expression becomes *n*:0:*n*. Sequence analysis of the 16S rRNA confirms the monophyly of all cultivable spirochetes (9). The genera, as determined physiologically and morphologically (10), are correlated with 16S rRNA sequences (9). The five genera of complex symbiotic spirochetes, with crenulations, cytoplasmic tubules, structured coats of the membranes, polar organelles, etc. are not cultivable (11). Morphometrics in uncultivable spirochetes provide the basis for taxonomy (12). Spirochete-cell diameter is usually constant for any strain, whereas physiological conditions that inhibit growth tend to increase cell length. Spirochete diameters vary from 0.09 to at least 3 μm and lengths from 3 to 500 μm. Pathogenic spirochetes associated with syphilis and Lyme disease, respectively, include *Treponema pallidum* (*n* = 1–3, transmitted sexually) and *Borrelia burgdorferi* (*n* = 7–15, borne by ticks, *Ixodes dammini, Ixodes pacificus, Ixodes ricinus*, and *Ixodes persulistus*).

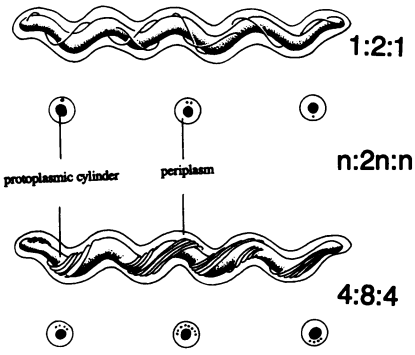

FIG. 1. Spirochete structure showing flagellar arrangement. Drawing was by Christie Lyons.

Morphometrics of Ehrenberg's uncultivable "type species" *Spirochaeta plicatilis* (diameter 0.75 μm, ≈20 flagella) are unavailable (14). No other "large" spirochetes (>0.5 μm in diameter) are free-living. Except *S. plicatilis*, large spirochetes are in digestive organs of animals—e.g., *Cristispira*, with only a spirillum and a mycoplasm, inhabits the style of molluscs (6, 13). Hindguts of wood-eating cockroaches (*Cryptocercus*) and termites (rhinotermitids, kalotermitids, and hodotermitids) harbor distinctive large spirochetes in great profusion. None have been cultured despite many attempts—e.g., Noguchi (2) and Breznak (13). Placement into anoxic media may prolong survival; yet within hours of removal symbiotic spirochetes die. *T. pallidum* responds to exposure by immediate death (15). Spirochetes reproduce by transverse binary fission. No developmental cycle has been documented for any spirochete.

METHODS AND MATERIALS

Samples were collected from three laminated intertidal microbial mats containing the filamentous cyanobacterium *Mi-*

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‖Shockingly, confusion concerning the identification of spirochetes, especially the causative agent of syphilis (*Treponema pallidum*), persists even among scholars who should be better informed. This recent book exacerbates the problem: "Syphilis has long fed on an hysterical panic that has ill-served the cause of prophylaxis . . . . Nowadays, by contrast, syphilis feeds on the carefree disdain of the general public. Can penicillin vanquish it? Of course, but one still has to know that one is contaminated. **The treponema is a tiny fragile thing, a vulgar protozoan, not even a virus.** But this fragility, which has made it so far impossible to culture *in vitro* and thereby gain a sufficient understanding of its modes of operation, assures its survival" (boldface type is our emphasis).

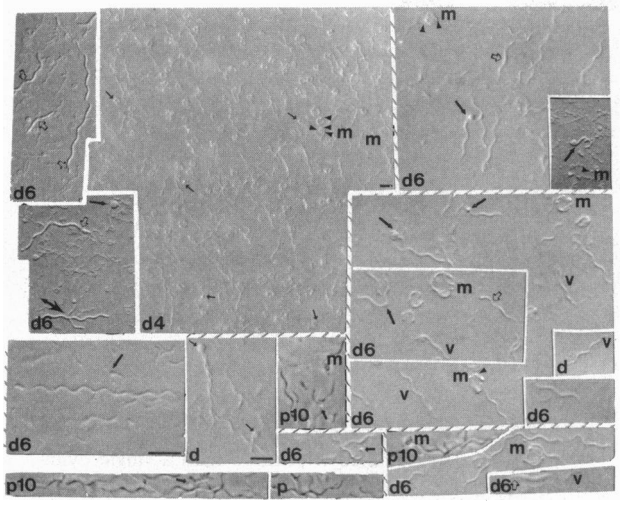

FIG. 2. *Spirosymplokos*, (s) live, with different objectives (p, phase-contrast light micrograph; d, differential interference-contrast micrograph; 4, ×140; 6, ×220; 10, ×350 original magnification). Open arrows, composite structure; solid arrows, round bodies and swellings; arrowheads at m correspond to membranous swellings in Fig. 5 *C* and *D*. At double-headed arrow smaller spirochete is attached to larger spirochete. (Bars = 10 μm.)

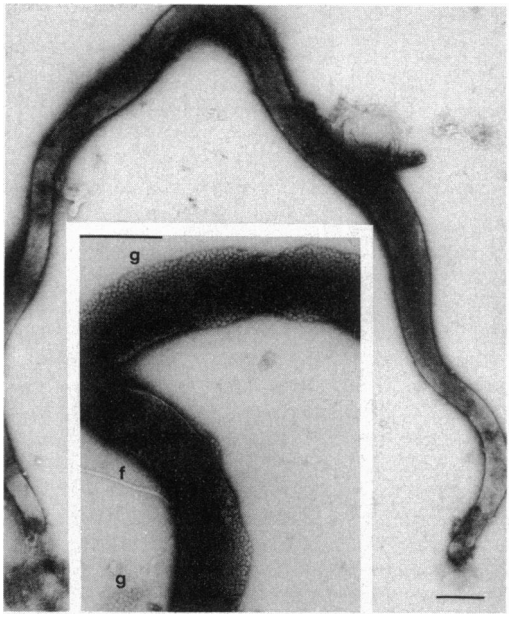

FIG. 3. *Spirosymplokos*, negative stain. (*Inset*) Higher magnification. g, Granules; f, flagella. (Bars = 1.0 μm.)

crocoleus cthonoplastes from Spain (16) and Mexico (figure 1, site 1 in ref. 17 and ref. 18). The best were consistently obtained at the Alfacs Peninsula of the Ebro delta (16). Enrichments were made by adding 1-cm^3 samples of all mat

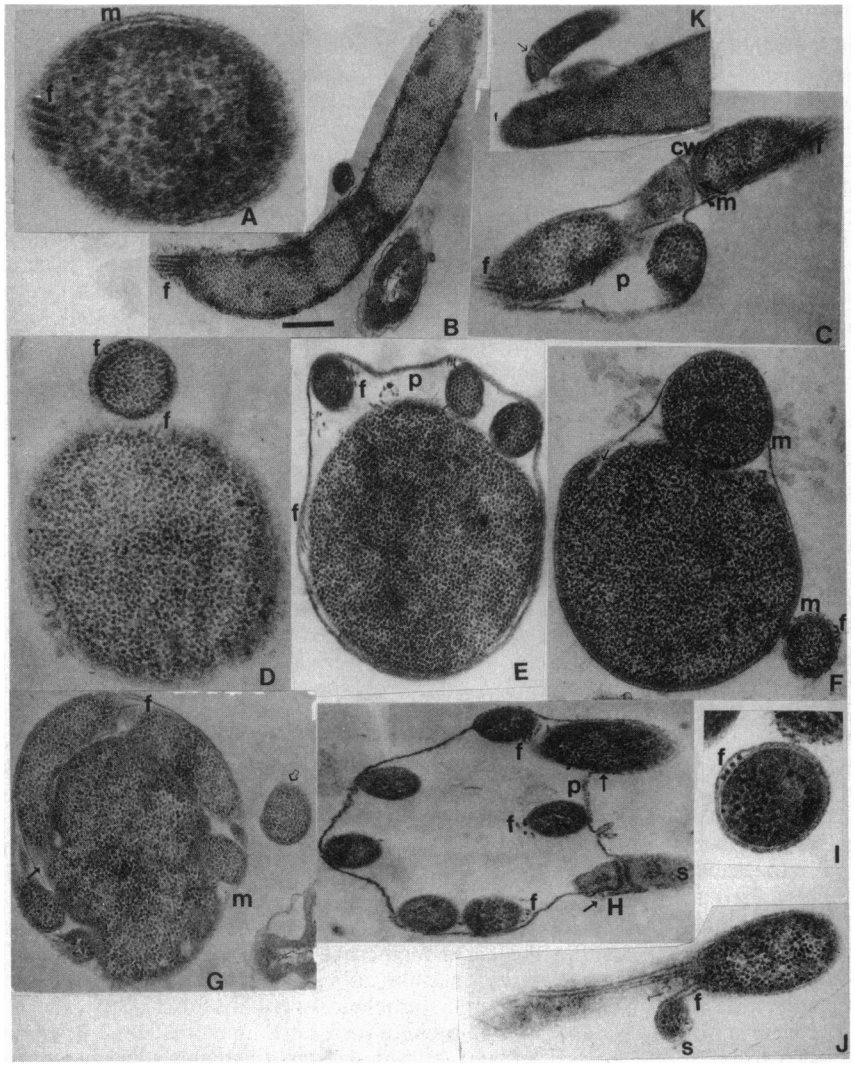

FIG. 4. (*A*) Composite structure; m, membrane. (*B*) Cytoplasmic granules may be continuous with components of the periplasmic flagella (f). (*C*) Formation of new cross walls (*cw*) inside common periplasm (p). (*D*) Small granulated spirochete with five flagella (f) in common periplasm of large protoplasmic cylinder. (*E*) Four protoplasmic cylinders in common periplasm (f, flagella; p, periplasm). (*F*) Granulated spirochetes both inside and outside outer membrane (m; arrow, cytoplasmic cleavage; f, flagella). (*G*) Continuity between large and small protoplasmic cylinders (arrow) in transverse section. Smaller spirochete with granulated cytoplasm at right (open arrow). (*H*) Smaller spirochete (s) apparently emerging (or entering?) through outer membrane (f, flagella). (*I*) Small spirochete recovered from nearly dry mat material. (*J*) Smaller diameter spirochete (s) interpreted to be developmentally connected by the flagella (f) to larger one. (*K*) Small granulated spirochete (cross wall at arrow) attached to the large variable-diameter one.

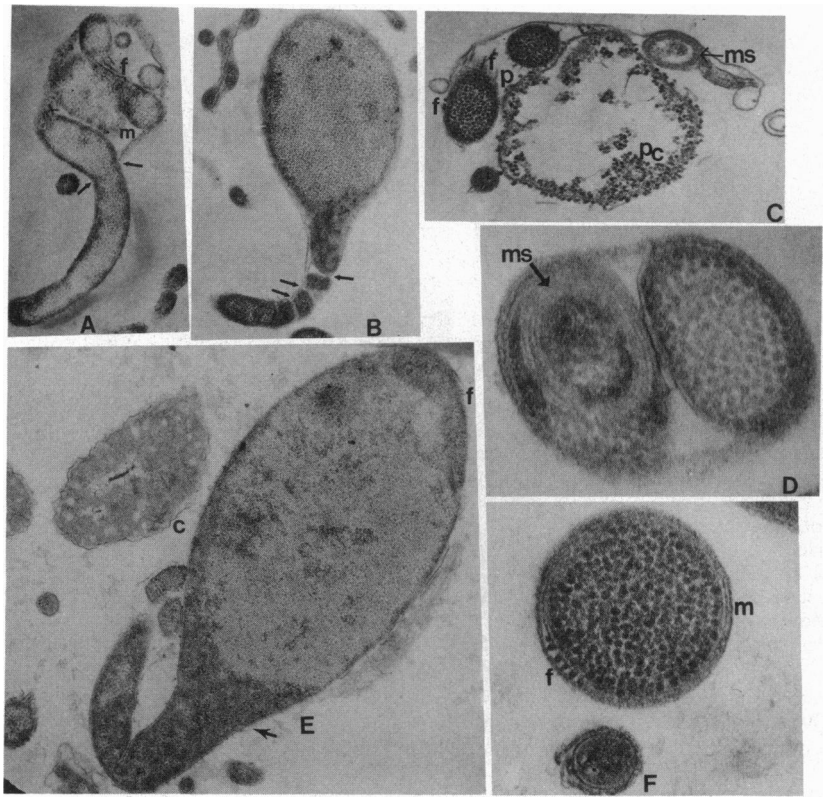

FIG. 5. (*A*) Flagella (f) and granules associated with more than a single cylinder, one with its own membrane (m) constricted. Spirochete retracting (or less likely, emerging) at arrows. (*B*) "Budding-bacteria"-like swellings and development of membranous structures, protoplasmic cylinder cleaved, arrows. (*C*) Membranous structure (ms) in disintegrating protoplasmic cylinder (pc) in its periplasm (p) probably just before release. (*D*) Membranous structure (ms, arrow) in small granulated spirochete. (*E*) Variable diameter (arrow) of large spirochete (f, flagella; c, *Chromatium*-like phototroph). (*F*) Spirochete membrane (m) thickening.

layers and underlying mud into BA2rif medium (cellobiose/yeast extract/trypticase peptone/antibiotic rifampicin/80% seawater; see refs. 19 and 20 for details). Inoculated tubes were incubated at 22, 25, or 31°C. Spirochete behavior was observed and recorded with a Sony U-Matic videocamera mounted on a Nikon Microphot. Concentrated by centrifugation, spirochetes were prepared for thin section or negative stain transmission EM analysis (5, 12). As detailed (20), samples fixed in 1.25% glutaraldehyde were washed, centrifuged, postfixed in osmium tetroxide, rewashed, dehydrated, and embedded. Stained sections were examined at 80 kV with a JEOL-CS electron microscope.

RESULTS

After inoculation with fresh field samples (6–9 days) in about one-third of the tubes spirochetes "bloomed" (i.e., developed population densities of three to six large spirochetes per field at ×400 magnification). At the height of the bloom, preparations were made for light, video, and EM. Invariably small spirochetes, rods, cocci, and spirilla grew. Sporadically blooms developed of a *Tricercomitus*-like mastigote or the anaerobic ciliate *Trimyema*.

The large spirochetes were easily seen in phase-contrast at ×200 original magnification (Fig. 2). Only those from Spain were studied in detail. With 1 cm^3 of original microbial mat sediment, growth and transfer of the spirochetes was extended for >6 weeks. Boiled, autoclaved, or filtered mud extracts did not suffice. On transfer to fresh BA2rif medium lacking mat, large spirochetes were outgrown by other bacteria. Some small spirochetes were isolated into axenic cultures, and mixed cultures were transferred indefinitely at room temperature or frozen (−80°C or −20°C). Large spirochetes, from seven excursions (August 1990; May and September 1991; February, May, July, and October, 1992), were seen in >40 samples. For at least 4 mo after collection of drying, but still damp, microbial mats placed in jars, spirochetes were grown in anoxic enrichments. Taken from 10 different mat samples, some 250 micrographs of at least 30 different specimens of large spirochetes were analyzed.

The large, loosely coiled spirochete, which swam with both smooth and jerky movements (negatively stained in Fig. 3) consistently had granulated cytoplasm (Figs. 4 and 5). Both uncoordinated and coordinated swimming occurred in the same spirochete: only a portion of the helix moved vigorously or movement occurred in two separated segments of the cell. Single large spirochetes also swam as a unit, for example, when seeking light of the microscopic field. When one end reached the darkened edge of the microscope field closed by an iris diaphragm, the spirochete changed direction moving toward the illuminated center displaying phototaxis (or possibly thermotaxis). Confirmed by videomicroscopy, behaviors were interpreted to be consistent with the composite structure in Figs. 2 and 4 *D* and *G*; also refs. 20, 21, and 23.

The ratio of the diameter of the protoplasmic cylinder to the diameter criterion 8 (figure 1*C* of ref. 12) was larger than any reported; for other morphometrics see ref. 20. From three to six flagella were inserted subterminally at each end. The cytoplasm was replete with dark granules in all protoplasmic cylinders, obscuring any nucleoids. Some of the 26 ± 6-nm-diameter granules extruded from the cells (ref. 20). The 26-nm-wide flagella were about the same diameter as the granules in >100 micrographs. The granules seemed continuous with the flagella (Fig. 4 *A* and *B*). In live and negative-stained cells large spirochete termini were tapered, and yet inside the periplasm of the smaller ones they were blunt (Figs. 3 and 4*C*), suggesting termini developmentally change as they grow. In each large spirochete, >1 and up to 16 granulated protoplasmic cylinders were present in nearly every transverse, oblique, or longitudinal section (Figs. 4 *D–G* and 5 *A–C*). Constant-diameter flagella, associated with both large (3.0 μm) and small (0.4 μm) protoplasmic cylinders were within the same membrane (Figs. 4 *C–E*, *G*, *J* and 5*C*). Rosettes, cytoplasmic tubules, bundles, and certain other features of large spirochetes were absent (12).

The same spirochete varied in diameter (Figs. 2, 3, 4 *G* and *K*, and 5 *B* and *E*). Similar small-diameter spirochetes were

found both inside and outside the outer membrane (Fig. 4 *D–G, I*). Continuity of large with small protoplasmic cylinders and several inside a common membrane is consistent with the idea that the variable-diameter spirochete is composite (Figs. 3, 4 *F* and *G*, and 5 *B* and *E*). Cross-wall products of cell division and cleaved cytoplasm suggest that small periplasmic spirochetes resulted from multiple fission (Figs. 4*G* and 5*B*; also ref. 20 and in ref. 21 figures 9–3 and 9–13). Live small spirochetes seem to be released through the membrane of the large ones. Small spirochetes, from one to three per cell, were seen attached to, perhaps emerging from (or entering?), large swimming ones, comparable to the micrographs of Figs. 2; 4 *F*, *H*, and *K*; and 5 *C* and *E*. That different-diameter spirochetes contained fully granulated cytoplasm (Figs. 4 and 5) and connections exist between the flagella of smaller and larger diameter spirochetes (Fig. 4*J*) support the idea that smaller spirochetes came from composite larger ones.

The large spirochetes became swollen on exposure to air (Fig. 2, Fig. 5 *B* and *E*). Some were videotaped as they actively withdrew their protoplasmic cylinders into the periplasm, a process captured in light (Fig. 2, solid arrows) and by EM (Fig. 5*A*). The onset of erratic, slower swimming, swelling, and withdrawal appeared developmental. Within a few hours while they continued to move, from one to four refractile bodies formed in nearly all. These became visible after the protoplasmic cylinders were withdrawn (**m** in Fig. 2). Refractile bodies prominent in swollen live spirochetes (Fig. 2, ref. 20) correspond to membranous structures in electron micrographs of Figs. 5 *C* and *D* and in ref. 20. Such behavior was not seen in desiccating cultures of *Spirochaeta* (*S. litoralis* or *Spirochaeta* sp. DE-1, refs. 19 and 23).

At all three sites in >20 trials [Spain (16), Laguna Figueroa (17), and Guerrero Negro, Mexico (18)] the spirochetes came only from laminated *Microcoleus* mats. Granulated-cytoplasm spirochetes were in contact with *Chromatium*-like cells in thin sections, suggesting they feed on photosynthate. Large spirochetes not yet studied were enriched from mats at Santa Pola (Alicante, Spain), Tenerife (Canary Islands, Spain), and Sippewisett salt marsh (Massachusetts) in which the phototroph *Microcoleus chthonoplastes* was underlain by purple sulfur bacteria (*Thiocapsa* sp., *Chromatium* sp., and others). Damp mats were adequate but large composite spirochetes were not retrieved from entirely dry samples.

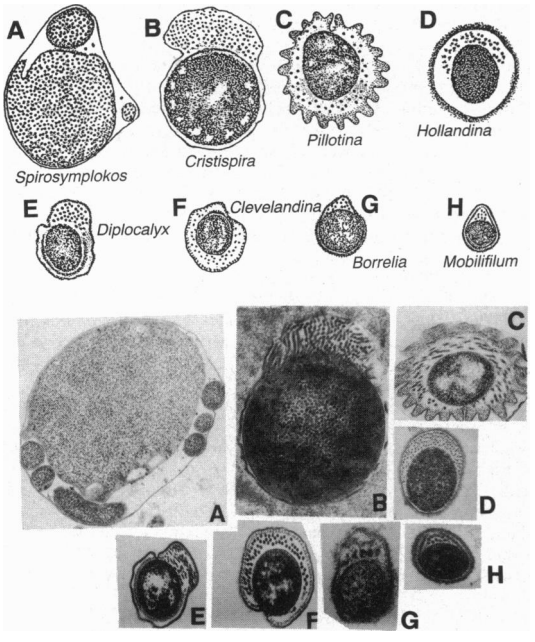

FIG. 6. Eight genera of complex spirochetes. Drawing by C. Lyons was based on text and Table 1. Transverse sections of eight genera are ordered by diameter size. *Cristispira* are from molluscs, and *Borrelia* are from mammals and ticks; other symbionts occur in isopteran digestive systems. Microbial mat *Spirosymplokos* and *Mobilifilum* only are free-living.

DISCUSSION

The morphometric description led us to introduce into the literature the Ebro delta large microbial mat spirochete as *Spirosymplokos deltaeiberi* (20). The generic name meaning braid or complex helix refers to composite morphology, the specific to where it was first found. It is compared with all 12 other spirochete genera in Table 1. An analytical drawing based on EM depicts *Spirosymplokos* with the other seven showing complex ultrastructure (Fig. 6). Only *Spirosymplokos* large spirochetes do not inhabit animal digestive organs.

Spirosymplokos by hypothesis undergoes morphogenesis: protoplasmic cylinder cleaves forming smaller spirochetes released from the parent. In response to air (oxygen, desiccation?) refractile bodies develop. Both the smaller and the larger protoplasmic cylinders (Figs. 4 *D*, *E*, *G*, *H*, and 5*A*) may provide source material for flagellar development. The paucity of flagella in the large cell raises questions: can so few flagella generate such active motility or might granules con-

Table 1. Comparison of morphology of all spirochete genera

Genus*	Habitat, characteristics	Number of flagella, range	Diameter, μm range and refs. for distinctive features†
Borrelia	Ticks, +, an, mi	7–15	0.2–0.5 (22)
Clevelandina	Termites, −, an	30–45	0.4–0.8 (11, 12)
Cristispira	Styles of bivalve molluscs, −, mi	100–300	0.5–3.0 (6, 13)
Diplocalyx	Termites, −, an	40–60	0.7–0.9 (11, 12)
Hollandina	Wood-eating cockroaches, termites, −, an	30–60	0.4–1.0 (11, 12)
Leptonema	Vertebrates, +, ae	1–4	0.1–0.3 (10, 24)
Leptospira	Vertebrates, +, ae, free-living	1–4	0.1–0.3 (10, 24)
Mobilifilum	Microbial mats, −, an	10	0.25 (25)
Pillotina	Termites, −, an	40–80	0.6–1.5 (11, 12)
Serpulina	Vertebrates, +, an	8–9	0.1–0.4 (9, 25)
Spirochaeta	Mud, +, an, mi, facultatively an	1–20	0.2–0.75 (14)
Treponema	Vertebrates, +, an	1–16	0.09–0.7 (5, 10, 26)
Spirosymplokos	Microbial mats	3–6	0.4–3.0 (20, 23, this paper)

*Transmission EMs of transverse sections and drawings based on these and many others are depicted in Fig. 6; Figs. 4 and 5 show distinctive morphology of *Spirosymplokos*. +, Some cultivable; −, uncultivable; ae, aerobic; an, anaerobic; mi, microaerophilic. Table was constructed from refs. 5, 6, 10–14, 20, and 22–27.

†Distinctive features are as follows: Lyme disease agent (*Borrelia*); chambered inner coat of outer membrane, sillon (*Clevelandina*); rosettes, flagellar bundle (*Cristispira*); thick outer coat of inner membrane, cytoplasmic tubules (*Diplocalyx*); developed outer coat of outer membrane usual, cytoplasmic tubules, polar organelle (*Hollandina*); cytoplasmic tubules, bent ends, gram + type basal flagella complex (*Leptonema*); leptospirosis agent (*Leptospira*); double outer membrane, polar organelle, flagellar bundle (*Mobilifilum*); crenulations, sillon, cytoplasmic tubules (*Pillotina*); swine-dysentary agent (*Serpulina*); vast group free-living: marine, freshwater, soil (*Spirochaeta*); syphilis, yaws agents (*Treponema*); composite protoplasmic cylinders, granulated cytoplasm, membranous bodies (*Spirosymplokos*).

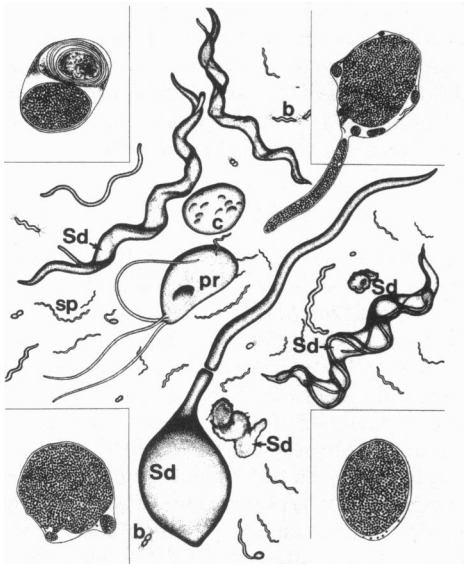

FIG. 7. *Spirosymplokos deltaeiberi* (Sd) reconstructed from live material and from micrographs as in corners of drawing. Variable-diameter large composite spirochetes release small ones. Swollen protoplasmic cylinders withdraw before formation of hypothetically viable membranous bodies. Members of community include *Chromatium* (c), anaerobic protists (pr), small spirochetes (s), and other bacteria (b). Drawing was by C. Lyons.

tain motility proteins? Of the spirochetes only *Cristispira* (6) has some granulated cytoplasm. Granules strewn on grids in negative-stained preparations (shown in refs. 20 and 23) are not fixation artifacts; whether these are related to flagellar components or to ribosomes (they are larger than typical 20-nm ribosomes) is unresolved. As membrane segregates growing cylinders (Figs. 4 *F*, *G*, and 5*B*), granule proteins may contribute to newly forming distally assembling flagella. Swellings (Fig. 2) correlated with the "budding-bacteria"-like appearance (Figs. 5 *B* and *E*) precede refractile body formation. The hypothetical developmental scheme as interpreted from life, videotape, micrographs of Figs. 4 and 5, and many not shown, is in Fig. 7. Cytoplasm in predatory prokaryotes differs from that of prey (28), and our micrographs were of vigorously growing cultures; the idea that small spirochetes inside parasitize the larger one is implausible.

The refractile, membranous bodies provide a morphological basis for possible oxygen and desiccation resistance. The transformations may relate (*i*) enrichability of spirochetes from desiccating microbial mats, (*ii*) the formation of spirochete round bodies, and (*iii*) the unpredictable appearance of spirochetes in tissues of syphilis and Lyme disease patients. Chronic spirochetoses symptoms and correlated motile bacteria often reappear after long dormancy periods (1). Although the explanation must also be immunological, the possibility must be reconsidered that symptom reappearance is related to spirochete differentiation; in culture round bodies may be abortive development stages (29).

Anoxygenic and oxygenic phototrophic bacterial mats are one of the oldest ecosystems on Earth. Mud spirochetes, aerotolerant anaerobic chemoheterotrophs that survive changing intertidal environments, are probably among the most ancient mat inhabitants. Ancestors of the large intestinal spirochetes most likely were mud-dwellers originally ingested with algal debris. That the rigors of littoral environments can be tolerated is consistent with an ancient history and early diversification of resistant spirochetes. Morphogenetic transformation in these fast-moving bacteria can be used as another argument that, in eukaryosis, undulipodia (cilia, sperm tails) evolved from spirochetes. Free-living spirochetes capable of responsive morphogenesis were the hypothetical ancestors of the now-intracellular microtubule/centriole-kinetosome system. The likely way in which, as motility symbionts, spirochetes literally insinuated themselves into *Thermoplasma* to become the eukaryotic cell lineage is detailed in ref. 21.

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